August 27, 2024

To whom it may concern,

I declare that the review process for the AOP report titled “AOP Report: Development of an adverse outcome pathway for deposition of energy leading to bone loss” and its accompanying AOPWiki entry (https://aopwiki.org/aops/482) was carried out according to OECD guidance for the scientific review of AOPs (OECD, 20211). All related documents are appended below. The review was conducted by the following committee members:

Review Manager:
Rex Fitzgerald

Reviewers:
Joshua Alwood
Leigh Gabel

Sincerely,

Jason O’Brien, PhD
Handling Editor, Environmental and Molecular Mutagenesis

OECD (2021). Series on Testing and Assessment No. 344: Guidance Document for the scientific review of Adverse Outcome Pathways. Organisation for Economic Cooperation and Development, Paris. Available at: https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm.

COACHES CHECKLIST AND REVIEW REPORT

ver. 2022-04-27

AOP Information

AOP number/title: 482, Deposition of energy leading to occurrence of bone loss

Author: Snehpal Sandhu, Mitchell Keyworth, Syna Karimi-Jashni, Dalya Alomar, Benjamin Smith, Tatiana Kozbenko, Robyn Hocking, Carole Yauk, Ruth C. Wilkins, Vinita Chauhan

Associated wiki page: *https://aopwiki.org/aops/482*

Compliance Reviewer Information

Name: Jason O'Brien

Organisation: Environmental and Molecular Mutagenesis

E-mail: jason.obrien@ec.gc.ca

Review Information

Date this checklist has been filled: 2023-06-02

Date of final draft PDF snapshot proposed for external review: 2023-06-02

AOP Coach Checklist and Final Review Report

General Observations and Recommendations of the Reviewer

- Technically, its two linear aops combined
- Several KEs and KERs are shared and some have been previously reviewed

KE ID	KE Title	Previously reviewed?	Which AOP?
1686	Deposition of Energy	YES	272
1392	Oxidative Stress	YES	17, 220
2066	Altered Signaling Pathways	no	
1825	Increase, Cell death	no	
2089	Altered Bone Cell Homeostasis	no	
2090	Increase, Bone Remodeling	no	
2091	Occurrence, Bone Loss	no	

KER ID	TITLE	ADJACENCY	Reviewed?	Which AOPs?
2769	Energy Deposition leads to Oxidative Stress	adjacent	NO	
2716	Oxidative Stress leads to Increase, Cell death	adjacent	NO	
2771	Oxidative Stress leads to Altered Signaling	adjacent	NO	
2842	Increase, Cell death leads to Altered Bone Cell Homeostasis	adjacent	NO	
2843	Altered Signaling leads to Altered Bone Cell Homeostasis	adjacent	NO	
2844	Altered Bone Cell Homeostasis leads to Bone Remodeling	adjacent	NO	
2845	Bone Remodeling leads to Bone Loss	adjacent	NO	
2846	Oxidative Stress leads to Altered Bone Cell Homeostasis	non-adjacent	NO	
2847	Energy Deposition leads to Altered Bone Cell Homeostasis	non-adjacent	NO	
2848	Energy Deposition leads to Bone Remodeling	non-adjacent	NO	
2489	Energy Deposition leads to Bone Loss	non-adjacent	NO	

AOP Coach Checklist and Final Review Report

Checklist

The following tables are checklists for the individual KEs and KERs and overal AOP

KE number, title: *1686, Deposition of Energy*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?	*272*			
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments: • *Will ask authors to define components during scientific review*				

AOP Coach Checklist and Final Review Report

KE number, title: *1392, Oxidative Stress*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?	***17 220***			
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?	*YES*			
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments:				

AOP Coach Checklist and Final Review Report

KE number, title: *2066, Altered Signaling Pathways*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?				*NO*
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			

Specific Comments:

- *Will ask authors to define components during scientific review*

AOP Coach Checklist and Final Review Report

KE number, title: *1825, Increase, Cell death*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?				*NO*
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments: • *Will ask authors to define components during scientific review*				

AOP Coach Checklist and Final Review Report

KE number, title: *2089, Altered Bone Cell Homeostasis*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?				*NO*
Has the KE been reviewed by EAGMST?				*NO*
If an existing KE is being adapted, have the previous authors been informed?				*X*
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			

Specific Comments:

- *Will ask authors to define components during scientific review*

AOP Coach Checklist and Final Review Report

KE number, title: *2090, Increase, Bone Remodeling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?				*NO*
Has the KE been reviewed by EAGMST?				*NO*
If an existing KE is being adapted, have the previous authors been informed?				*X*
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			

Specific Comments:

- *Will ask authors to define components during scientific review*

AOP Coach Checklist and Final Review Report

KE number, title: *2091, Occurrence, Bone Loss*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?				*NO*
Has the KE been reviewed by EAGMST?				*NO*
If an existing KE is being adapted, have the previous authors been informed?				*X*
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			

Specific Comments:

- *Will ask authors to define components during scientific review*
- *Regulatory significance is blank*

KEY EVENT RELATIONSHIPS

KER number, title: *2769 Energy Deposition leads to Oxidative Stress*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?	*YES*			
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?	*YES*			
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

AOP Coach Checklist and Final Review Report

KER number, title: *2716 Oxidative Stress leads to Increase, Cell death*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?	*YES*			
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?	*YES*			
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2771 Oxidative Stress leads to Altered Signaling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?	*YES*			
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?	*YES*			
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2842 Increase, Cell death leads to Altered Bone Cell Homeostasis*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

AOP Coach Checklist and Final Review Report

KER number, title: *2843 Altered Signaling leads to Altered Bone Cell Homeostasis*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

AOP Coach Checklist and Final Review Report

KER number, title: *2844 Altered Bone Cell Homeostasis leads to Bone Remodeling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

AOP Coach Checklist and Final Review Report

KER number, title: *2845 Bone Remodeling leads to Bone Loss*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2846 Oxidative Stress leads to Altered Bone Cell Homeostasis*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2847 Energy Deposition leads to Altered Bone Cell Homeostasis*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2848 Energy Deposition leads to Bone Remodeling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

AOP Coach Checklist and Final Review Report

KER number, title: *2489 Energy Deposition leads to Bone Loss*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

AOP Coach Checklist and Final Review Report

OVERALL AOP

Overall AOP	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Does the title of the AOP follow the correct convention (MIE or first KE leading to AO)?	*YES*			
Does the title of the AOP reflect its content/domain?	*YES*			
Is a graphical representation included?	*YES*			
Is it clear who the authors/developers of the AOP are? Contact information for one or more corresponding author(s) should be included.	*YES*			
Is the status of the AOP described?	*YES*			
Does the abstract concisely describe the main content of the AOP in a standalone manner?	*YES*			
Have prototypical stressors been identified for the MIE?	*YES*			
Has the regulatory relevance of the AO been described?	*YES*			
Is the domain of applicability of the AOP defined in accordance with the OECD AOP Handbook?	*YES*			
Is the level of support for essentiality of the KEs described and assessed in accordance with the OECD AOP Handbook?	*YES*			
Has consideration been given to the level of support for the calls on the Overall WoE and the Quantitative Understanding?	*YES*			
Specific Comments:				

REVIEWEWER #1:

Comments to Authors:

AOP Manuscript

Thanks for the opportunity to review the AOP. I focused my review primarily on bone remodeling and bone loss. In general, the AOP is well-written and includes a large body of scientific evidence to support the KERs. I note several areas for clarification below in both the journal article and wiki. Specifically, there appears some misreporting of study findings and incorrect descriptions of bone cell homeostasis.

1. Page 4, line 11. This sentence reads as if bone loss in post-menopausal women is due to the lack of mechanical loading due to microgravity. Suggest clarifying.
2. Page 5, line 47. Although some (but not all) animal studies may show decreased bone formation, human spaceflight studies indicate either no change in bone formation or an increase in bone formation *(see review by Stavnichuk et al., or primary research: Smith, S. M. et al. Bone metabolism and renal stone risk during International Space Station missions. **81**, 712–720 (2015), Smith, S. M. et al. Men and Women in Space: Bone Loss and Kidney Stone Risk After Long-Duration Spaceflight. J. Bone Miner. Res. **29**, 1639–1645 (2014)*. Most animal literature demonstrating decreased formation occurred in growing animals; mature animals models suggest no change in bone formation. *E.g., Smith, B. J. et al. Skeletal Unloading and Dietary Copper Depletion Are Detrimental to Bone Quality of Mature Rats. J. Nutr. **132**, 190–196 (2002).* It should be clarified here or in the Wiki that findings related to bone formation may differ based on life stage (e.g., growing or not).
3. Page 7, line 32. Is there a missing word in the sentence: “exposure to therapeutic has been”.
4. Page 14, line 52. “enhances osteoblasts” doesn’t make sense. Sclerostin downregulates osteoblast OPG production and decreases osteoblastogenesis, not enhances it.
5. Page 16, line 21. Suggest qualifying that current evidence “in animal models” demonstrate increased osteoclast number…
6. Page 18, line 39. Is it worth qualifying that most data were derived from “male” adolescent and animal models?
7. Page 21, lines 32-48. Re: bone resorption at the tissue level. The KE (2090) indicates calcium biochemistry. Human spaceflight studies typically assess calcium and phosphorus homeostasis, so would expect those findings to be included. Further, time-lapsed in-vivo micro CT imaging in animal models or high-resolution CT imaging in humans can quantify bone resorption, including eroded surface and bone resorption rate. Not sure whether studies have used these methods to evaluate resorption due to energy deposition, but the technique exists: Schulte, Friederike A et al. “In vivo micro-computed tomography allows direct three-dimensional quantification of both bone formation and bone resorption parameters using time-lapsed imaging.” *Bone* vol. 48,3 (2011): 433-42. doi:10.1016/j.bone.2010.10.007. Christen, Patrik, and Ralph Müller. “In vivo Visualisation and Quantification of Bone Resorption and Bone Formation from Time-Lapse Imaging.” *Current osteoporosis reports* vol. 15,4 (2017): 311-317. doi:10.1007/s11914-017-0372-1
8. Figure 2. Are the osteoclast and osteoblast sections flipped in the “altered bone cell homeostasis” sections?
9. Figure 7. Does the size of the KE in the circles have meaning? The legend indicates that size of the arrow does, but nothing is mentioned about the size of the KEs.

10. Supplementary Figure 1. Total number of records evaluated N=246 differs from what is indicated (e.g., N=107 extracted + N=75 other sources).

AOP Wiki

11. As per recommendations from American Medical Association, preferred usage is "White" in place of Caucasian. Flanagin, A., Frey, T., Christiansen, S. L. & Committee, A. M. of S. Updated Guidance on the Reporting of Race and Ethnicity in Medical and Science Journals. *JAMA* **326**, 621–627 (2021).
12. Spelling: "Further efforts could be directed to developing "mthods" that are able to assess bone resorption at the tissue level."
13. In the bone remodelling KE, 2090, an example states: "increased resorption by osteoclasts and increased mineralization by osteoblasts will increase the rate of bone resorption and decrease the rate of bone formation." However, increased mineralization by osteoblasts would increase the rate of bone formation, not decrease it. Please clarify.
14. Is there a reason why human spaceflight or analog studies were not included in the relationship 2844 – altered bone cell homeostasis leads to bone remodelling. Most human spaceflight studies indicate altered bone homeostasis due to substantial increases in biomarkers of bone resorption (e.g., CTx) with minimal change or increases in biomarkers of bone formation (e.g., BSAP, P1NP, or OCN).
15. Re: 2844 – it is an important qualification that most animal studies demonstrating altered bone homeostasis as defined by an increase in osteoclast activity and decrease in osteoblast activity are in growing animals. Mature animal models tend to suggest no change or an increase in bone formation (e.g., Hui et al 2014, Wright et al 2015). If the KER evidence is based on increased bone resorption and decreased bone formation, it would appear the evidence should be higher for juvenile life stages. Or perhaps the KER evidence is based on altered bone homeostasis since increased bone resorption on its own without decreased formation can still result in decreased mineral apposition rates?
16. Similar comments for relationship 2845 re: why were human spaceflight or analog studies not included in the KER (e.g., Stavnichuk et al)?
17. Re: relationship 2849: "Short duration flights (<30 days) led to decreased bone density up to 10%, which could be due to an early onset of increased resorption and late onset of increased formation (Stavnichuk et al., 2020)." This sentence is incorrect and is not supported by the review by Stavnichuk et al. Changes in BMD are usually not detected in short duration flights.
18. Evidence assessment: KE 2090 → AO2091: the evidence supports bone loss as an imbalance between resorption and formation due to increased bone resorption. Less evidence supports a decrease in bone formation in mature animal models or in humans.
19. Evidence assessment: Known modulating factors: "Estrogen, which is lower in old age, decreases osteoclast activity and increases osteoblast activity by inhibiting the production of interleukin (IL)-6 in osteoblasts (Pacheco and Stock, 2013)." The role of osteoclasts and osteoblasts appears reversed.
20. Re: consideration for potential applications of the AOP: "An uncertainty in the bone remodeling KE is that changes to the rate of resorption are not directly determined and are instead assumed based on changes to the bone formation rate and bone volume". It's unclear why calcium biochemistry studies were not included in the review since they are listed in the method of measurement.

REVIEWER #2

Reviewer Response to AOP Wiki on Energy Deposition and Bone Loss

I have reviewed the article entitled *Development of an Adverse Outcome Pathway for Deposition of Energy Leading to Bone Loss* and the AOP #482 "Bone Loss Snapshot" supplement by S. Sandhu, et al., which constructs a SME- and literature-informed AOP (shown in their Figure 1) with strength of connections (shown in their Figure 7) for bone loss following energy deposition after exposure to ionizing radiation with applications to radiotherapy and spaceflight. These contributions total ~173 pages. As described by the authors in Section 4 and Supplementary Figure 1, approx. 1865 articles were filtered down to 246 included references, representing a tremendous and laudable amount of detailed and formalized assessment and synthesis.

The AOP #482 is present on the website AOPwiki.org under a copyright designation as all rights reserved requesting permission for re-use from authors. Additionally, this website lists the primary authors (many from the Consumer and Clinical Radiation Protection Bureau of Health Canada) and consultants (from the USA and Japan). Of note, the submitted manuscript does not differentiate between authors and consultants; all are listed as authors.

According to the Instructions for Scientific Reviewers, the manuscript should have already undergone a Preliminary Compliance Review (hence, is "structurally compliant") to adhere to OECD's conventions for AOP development. Thus, additions of new KEs by reviewers are not recommended.

Overall, the article is well-written and conveys a high-level of scientific quality, but addressing the following critical points could improve the framing, interpretation and limitation statements of the primary material, and diversity and weight of evidence – specifically, including additional references and discussion of the broader AOP/KE landscape.

<u>Specific Questions on the AOP – please address for reader clarity</u>

1) Is it worth referencing and discussing sibling AOPs involving energy deposition in the Introduction or Discussion to show cross-organ importance, to show how the bone AOP fits into the broader AOP context, and/or to leverage findings from these AOPs to increase weight of evidence?
    a. Specifically, AOP #483 (*learning memory effects from energy deposition*) and AOP #470 (*vascular remodeling from deposition of energy*).
    b. Additionally, AOPs 327 through 330 (*Excessive ROS production leading to mortality*).
2) Page 12 and Figure 1 and Tables II and III, regarding the definitions of *Adjacent* (solid line) vs. *Non-adjacent* (dashed line) KEs, can you include a more formal definition and description of function for classification? It is not clear in the body of the manuscript, especially on page 19 (Uncertainties and Knowledge Gaps) where detailed discussions take place of adjacent KEs. Perhaps simply cutting/pasting the definitions used in the Figure 1 caption and minor additions may suffice.
3) Beginning in Section 5, page 12, the authors discuss a sequence of events following energy deposition leading to altered signaling and tissue changes that are also summarized in Figures 1, 2, and 7. However, upon review of the broader KE landscape and evidence base, it appears additional relevant KEs are neither included, nor discussed, for this particular AOP. Do additional KEs merit discussion to define the broader KE context or for future KE edits to

the AOP? The below items 4) and 5) identify some of the additional KEs for consideration to be discussed.

   a. Additionally, in the manuscript's content on Section 5, page 12, evidence from (Kondo et al. 2010) in the article's Figure 4 and 5 show a more complex timecourse than as described by this manuscript showing time-varying sequences of ROS, apoptosis, and lipid peroxidation contingent on dose of gamma-rays. As animal studies cannot capture all data from all timepoints, we don't know when these outcomes peak. My main point is that lipid peroxidation increases from acute response (day 0 and day 3) to 10 days post-exposure for 1 Gy and 2 Gy. Hence, this type of oxidative damage marker could be the result of a secondary insult (~weeks after exposure) that is not captured in this AOP manuscript's discussion of events on page 12.

4) Specifically for KE1392, does this KE include or interact with other key events (KEs) pertinent to free radical processes listed below, some of which are discussed on page 12 and nicely summarized/illustrated by (Nathan and Ding 2010)? In other words, are there sub-nodes or critical additional nodes that should be identified/discussed?
   a. Should MIE/KE 257 for ROS production be discussed?
   b. Should nitrosative stress (KE1632) be mentioned with "RONS" in the manuscript?
   c. Should KE 177 mitochondrial dysfunction and/or KE1170 mitochondrial membrane potential be discussed?
   d. Should lipid peroxidation be discussed (KE1445)?
   e. Should oxidation to nucleic acids be discussed (KE1608 / 1634)?
   f. Should protein oxidation products or oxphos be discussed (KE1767 / KE1477)?
5) Looking at downstream events, should the following KEs be discussed?
   a. Should KE1492 (tissue resident cell activation) and KE 1494 (Leukocyte-lineage: Monocyte-Macrophage-Osteoclast) – i.e., osteoclasts fit these bills – be discussed?
   b. Should KE 1493 and 2097 (pro-inflammatory mediators) be discussed? Evidence appears in these papers, for example,
      1. Tnf and MCP-1 from (Alwood et al. 2015)
      2. Tnf, IL-1beta, IL-6 from (Willey et al. 2011)
      3. Tnf levels from (Little-Letsinger et al. 2021)
      4. Tnf response (Shimizu et al. 1998)
   c. Should Nrf2 and the downstream antioxidant response element be discussed (KE 1417)? Here are some articles on Nrf2 and bone:
      i. Review articles (Sun et al. 2015; Han et al. 2022; Che et al. 2023)
      ii. Effects from loss of Nrf2 (Rana et al. 2012)
      iii. Bone biomarkers from Nrf2-/- mice following spaceflight (Suzuki et al. 2022)
      iv. Irradiation induced changes in Nrf2 in marrow (Liu et al. 2019) and (Schreurs et al. 2016)

<u>Specific Questions on the modified Bradford Hill Criteria</u>

6) The authors use a "*modified* Bradford Hill criteria" framework (Becker, et al, 2015 and illustrated in Figures 4 and 6). In Section 5, the authors state "**Biological Plausibility** is the highest form of evidence…". Consider adding some discussion on the definition of "highest form" to clarify this statement's intended meaning. Does this mean strongest or leading/initial form of evidence? For example, from a mechanistic point of view, this statement doesn't seem accurate if highest form is

interpreted to convey the *weight* of empirical evidence. Some added description will help the reader.

7) To my reading, Bradford Hill criteria were developed for epidemiological data synthesis where, outside of clinical trials, direct testing of cause and effect in humans is not feasible. How are mechanistic interventions, especially in animals, treated/accounted for in this selected Bradford-Hill framework? The authors state that countermeasures are used for assessing attenuation of upstream KEs and downstream normal biological status (Essentiality criteria, page 17) – more procedural detail added here could be helpful to the reader. Is it worth discussing the pros and cons and limitations of the selected modified Bradford Hill criteria for the animal/mechanistic datasets included?

<u>Comments on the Framing of the AOP #482</u>

8) Much of the irradiation evidence originates from animal models. It is recommended to increase the # of human cohort studies to increase the weight and strength of evidence. An example of current and very relevant work: have the authors consulted with the lead(s) (John D. Boice, Vanderbilt) of the Million Worker Study of radiation workers (Boice 2022) to discuss skeletal outcomes? If not, this is highly recommended to consider this or other non-radiotherapy human-cohorts that includes radiation exposure (i.e., low dose, chronic exposure).
9) <u>Structure of manuscript</u>: In terms of applicability to a broad human population, the authors *could* consider adjusting the focus of the AOP and discussion on occupational or cancer radiotherapy primarily (i.e., a relatively large-sized cohort) and have a conditional case or modulating factor for spaceflight (i.e., a small-sized cohort). Right now, these weightings seem inverted, especially considering inclusion of spaceflight data and simulated weightlessness data resulting from rodent models.
10) In the introduction of bone biology, it is highly recommended that the authors introduce cancellous/trabecular versus cortical bone compartments and discuss how radiation effects may present uniquely in time, geometry, and biology in each compartment following exposure. A few references:
    a. In rodents, trabecular effects from irradiation (Kondo et al. 2010).
    b. In rodents, cortical effects from irradiation are potentially more subtle and dependent on dose (Lloyd et al. 2008; Wernle et al. 2010; Oest et al. 2015; Sugimoto et al. 1991).
11) Consider including review articles for use in introducing topics that are well-developed in the literature:
    a. For bone cell introductions:
        i. ASBMSR Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 9th edition, misc chapters in Section I
    b. For radiation effects on bone and bone cells:
        i. (Donaubauer et al. 2020)
        ii. ASBMR Primer 8th and 9th Primers: (Wright 2018; Willey 2013)
    c. Mechanistic formulae to model bone turnover
        i. (Gerhard et al. 2009), (Boaretti et al. 2023), (Ayati et al. 2010)
    d. ROS/RNS/RONS and the skeletal system

i. (Marques-Carvalho, Kim, and Almeida 2023; Bartell et al. 2014; Almeida et al. 2009; Manolagas and Almeida 2007; Almeida et al. 2007)
ii. (Le Nihouannen et al. 2010; Agidigbi and Kim 2019).

12) Late in the manuscript - on page 20 - the authors state "Mature bones are relatively radioresistant". Overall, however, the paper fails to differentiate *bone* as relatively radioresistant and *marrow* - the source of stem and progenitor cells for the skeleltal system – as *highly* radiosensitive. It is recommend to introduce and discuss tissue sensitivity of the integrated skeletal system and weighting factors for dose equivalent calculations (ICRP 2007) and to introduce much earlier in the manuscript.
    a. With a large bolus dose, marrow can undergo acute cell killing and subsequent repopulation or outright marrow cellularity depletion/ablation. For example, see (Turner et al. 2013), (Cao et al. 2011), (Green et al. 2012), (Kim et al. 2014), (Tatara and Monzen 2023), and (Suva et al. 1993).
    b. Smaller doses do not induce the same level of acute cell death in marrow, hence is more easily recoverable by stem cell proliferation and differentiation (Otsuka et al. 2008) (Greenberger and Epperly 2009), though late effects are possible (Chatterjee et al. 2012), (Rastogi et al. 2012).
13) Description and framing of dose levels and bounds are critical to convey to the audience of this manuscript, both in terms of spaceflight dose and radiotherapy dose – which seems to be currently missing from the manuscript. In terms of spaceflight, doses range widely from ISS missions to lunar missions and to Mars missions (Hassler et al. 2014). The data from animal studies shows a threshold dose eliciting an effect (approx. 50 cGy of heavy ions (Yumoto et al. 2010),(Alwood et al. 2017) or 100 cGy of ^{137}Cs (Kondo et al. 2009), (Alwood et al. 2012) or proton (Bandstra et al. 2008)), which also hint at an LET effect on threshold dose. In these studies, lower doses had no effect on bone structure. Thus, how is the null hypothesis incorporated in the AOP at low dose and dose rates? At the other extreme, an upper bound of dose be introduced with appropriate conditions (e.g., focal, fractionation), with considerations for acute radiation syndrome, morbidity, and tissue necrosis/marrow ablation? In other words, bounding the dose ranges and/or energy imparted (LET) seem critical to framing the AOP properly. It is not one size fits all for *irradiation* effects on bone, which is how the manuscript currently comes across. Some potential citations:
    a. Acute dose range - (Donaubauer et al. 2020) Figure 1
    b. Protracted / fractionated dose range – (Richardson et al. 2022)
    c. Current mission and career dose limits for astronauts, *Standard 4.8 Space Permissible Exposure Limit for Space Flight Radiation Exposure* (NASA 2022)
14) For spaceflight or simulations using rodents, specifically, it is recommended that the framing be addressed throughout and especially on **Modulating Factors** on page 18 to include some items in the list below. Examples of articles supporting Modulating Factors for radiation, include evidence for dose response above a threshold, LET response, and osteoprogenitors proliferation and differentiation potential following radiation exposure (Alwood et al. 2012), (Alwood et al. 2017), (Bandstra et al. 2008), (Lima et al. 2017). A suggested list of Modulating Factors for irradiation response in the skeleton:
    a. Species and Strain of animal
    b. Age at exposure
    c. Anatomic location and compartment (cortical/cancellous) of the skeleton
    d. Diet: dietary countermeasures against irradiation should be discussed in more detail (e.g., absence/presence of radio-protectors like phytoestrogens; omega 3; Dried Plum)

i. Dietary dried plum protects against low-LET gamma and high-LET effects (Steczina et al. 2020)
ii. Omega 3 vs 6 diet comparison (Little-Letsinger et al. 2021)

e. Additional pharmaceuticals/countermeasures should be added on page 18 and identified as a modifying factor: e.g.,
   i. Bisphosphonates (Willey et al. 2010), (Keenawinna et al. 2013)
   ii. P7C3 (Wei et al. 2023),
   iii. The antioxidant alpha-lipoic acid (Kondo et al. 2010) and SOD (Alwood et al. 2017)
   iv. PTH (Oest, Mann, et al. 2016; Oest, Gong, et al. 2016)
f. Exercise as a countermeasure, e.g., (Shirazi-Fard et al. 2015), (Govey, Zhang, and Donahue 2016)
g. Reference space-mission type (ISS mission differs from planetary orbital differs from planetary surface operations) (Hassler et al. 2014)
h. Total body irradiation or focal irradiation
i. Exposure / Fractionation schedule
j. Dose rates
k. Radiation type and quality
   i. Linear energy transfer LET (Durante and Cucinotta 2011)
   ii. Track structure (Plante and Cucinotta 2008)

Additional Evidence to consider for inclusion within the AOP

15) Discussion of irradiation and mitochondrial role/function in the skeletal system are not included. The following articles offer two examples:
   a. A transgenic mouse study which expresses catalase antioxidant in mitochondria (mCAT) and treating mouse with combined unloading and irradiation to mimic spaceflight conditions – fundamental to free radical hypothesis (Schreurs et al. 2020). The animals still experience bone loss.
   b. Article investigating fractionated irradiation and sirt-3 in osteocytes (Richardson et al. 2022).
16) Cell death pathways, including apoptosis, necrosis, and autophagy are discussed on page 15, presumably primarily for dividing stem and progenitor/precursor cells in the marrow and also for differentiated cells in the osteoblast/osteocyte lineage. It does not appear that *senescence* is mentioned in the manuscript body (though at least 15 titles of references state *senescence*); A thorough discussion of Senescence Associated Secretory Phenotype (SASP) is neither introduced, nor developed, yet literature shows SASP, especially from *in vitro* studies to play a role in the cellular irradiation response at high doses, pertinent to tissue aging (Coppe et al. 2010; d'Adda di Fagagna 2024) (Kumar et al. 2022). *In vitro* work can be specific to osteocytes (Wang et al. 2021) and other bone-related cell types, including marrow HSCs (Wang et al. 2006) and MSCs (Bai et al. 2020).
17) Irradiation effects on bone cell replenishment from marrow precursors or progenitors is not specifically introduced/discussed. The following articles should be reviewed for potential inclusion:
   a. Effects of radiation dose and LET on growth potential of marrow after irradiation
      i. Stem cell pool depletion (Green et al. 2013; Green and Rubin 2014; Green et al. 2012) and oxidative damage (Cao et al. 2011)

ii. Oxidative stress and alpha-lipoic acid (Kondo et al. 2010) and SOD (Alwood et al. 2017) countermeasures for low-LET gamma irradiation
iii. Short-term effects following high-LET particulate irradiation (Yumoto et al. 2010)
iv. Dietary dried plum countermeasure against low-LET gamma or high-LET particulate irradiation (Steczina et al. 2020)

18) Acute and transient *stimulatory* effects of osteoblast lining cells has been found – this subtle evidence is not introduced in background or discussed in other sections (e.g., page 16 histological sections):
    i. Acute stimulation of osteoblast lining cells strongly correlated with marrow depletion (Turner et al. 2013).
    ii. 10 cGy stimulation of osteoblast lining cells (Karim and Judex 2014).
    iii. Apparently positive structural effects of low-LET irradiation (Bokhari et al. 2019).
    iv. Irradiation-induced increase in mineral apposition in cancellous tissue of the tibial metaphysis (Willey et al. 2010).
19) The full temporal complexity of responses in osteoclast cells is not captured in the background or later section discussions (e.g., page 16 histological discussions). In my view, shortly after irradiation exposure, there is an acute co-stimulation of osteoclasts along RANKL-induced and inflammatory pathways, yet, oppositely, there is indication of late inhibition of osteoclast differentiation and bone resorption:
    a. For radiotherapy doses, (Oest et al. 2015) is cited, but needs more discussion to capture the temporal effects of # of osteoclasts in the distal femoral metaphysis and epiphysis and late cortical thickening. Additional work from this group (Wernle et al. 2010) shows bone structure outcomes showing temporal variation for cancellous and cortical in distal femur.
    b. (Zhai et al. 2019) reports varying osteoclastogenic potential based on single or fractionated radiotherapy doses.
    c. (Alwood et al. 2015) reports temporal gene expression of osteoclastogenic and inflammatory factors in marrow following irradiation.
    d. (Willey et al. 2011) reports temporal osteoclast and inflammation markers following irradiation.
    e. (Wei et al. 2023) shows countermeasure against osteoclast induced bone resorption.
20) Irradiation effects on osteocytes are not discussed in depth. The first mention of osteocytes is not in the abstract, but rather in the middle of page 6. The following articles could be considered for inclusion to deepen this important area of knowledge:
    a. (Sugimoto et al. 1991), (Sugimoto et al. 1993), (Takahashi et al. 1994): Rabbits receiving focal electron irradiation at high doses show complex temporal effects, including tissue-level, marrow and osteocyte viability, and vascularity & haversian canals effects.
    b. Combined partial weight bearing and ^{28}Si irradiation induction of SOST-positive osteocytes (Macias et al. 2016) which reduces bone formation process.
    c. Additionally, remodeling of the lacunar space by osteocytes is not mentioned. Much is unknown about this process relating to irradiation, but there is some evidence of osteocytic osteolysis following spaceflight (Blaber et al. 2013) or simulated weightlessness (Lloyd et al. 2014).

21) Adipocyte infiltration into the marrow is not discussed, though much evidence exists (Ewing 1926; Bond, Fliedner, and Archambeau 1965) (Guerra et al. 2018; Costa and Reagan 2019; Nakashima et al. 2024), (Wei et al. 2023), (Chandra et al. 2017). Adipocytes could contribute to the pro-inflammatory milieu in marrow following irradiation.
22) Page 17: *In vivo* studies of abscopal effects - where bone loss is observed outside of the irradiated area - are few in #, but mechanistically are important to acknowledge. At least one uncited article shows focal irradiation of the gut having effects on bone (Jia et al. 2011). Another shows effects in a contralateral, unirradiated limb (Wright et al. 2015). EG Wright's work shows macrophages as critical links in abscopal effects in marrow (Rastogi et al. 2012), (Burr et al. 2010).
23) Bone matrix changes related to radiation and oxidative stress, like Advanced Glycation End-Products (AGEs) are not discussed, which could fit under ROS of protein oxidation KEs 257 and 1767. Some examples of relevant articles include:
    a. (Pendleton et al. 2021; Mandair et al. 2020; Gong et al. 2013; Oest and Damron 2014; Oest, Gong, et al. 2016)

<u>Minor suggestions for edits</u>

24) Page 5, Background: change "constant remodeling" to "continual remodeling", as remodeling rate is always present, but not a constant rate.
25) Page 6, "temporary anatomical structures" could cite (Jilka 2003)
26) Page 7, use of "evolve" to describe osteocytes. It's recommended to change this to "terminally differentiate"
27) Page 13, clause "through upregulation of RANK-L production in osteoblasts" could be appended with "osteoblast-lineage cells" as osteocytes should not be excluded.
28) Page 14, clause "and enhances osteoblasts by subsequently reducing OPG" should likely be "osteoclasts".
29) Page 15, regarding the clause "Due to inability of scavengers (phagocytes) to reach osteocytes", isn't this one function of osteoclastic bone resorption, especially in trabecular region and near cortical surfaces? Consider modifying this clause for accuracy.
30) Page 16, authors use "dysregulated bone remodeling" clause twice in this paragraph. Please review for revision.
31) Page 16, clause "promoting bone matrix resorption back into the bloodstream" is somewhat confusing (and also used in following sentence). Consider "bone matrix transport" instead of resorption.
32) Page 18, check logical use of "and" in lists of antioxidants and lists of transgenic animals. An "or" clause may be more appropriate.
33) Page 24, Potential Applications, the leading sentence "The present qualitative AOP was developed…" is important and frames the organization of everything prior. Can varieties of this sentence be inserted in additional places like the abstract and sections 1 and/or 3? It feels buried at this location.
34) Page 25, clause "risk assessment strategies", consider citing (Orwoll et al. 2013).
35) Page 66, Figure 2, "Tissue" box: the clause "shift to rod-like geometry", consider changing to "more rod-like" and that is only for trabecular tissue (see comment on cortical vs cancellous compartments above).

Citations in this review – for cross referencing and for consideration of inclusion in the "Collection of Evidence for AOP Building"

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Responses to Reviewers comments

Thank you for your thoughtful review of our adverse outcome pathway (AOP) manuscript and associated documents. We appreciate the time and effort you have dedicated to providing very constructive feedback. We have reviewed the comments and where appropriate have addressed them as outlined below. Please note the page and line numbers correspond to the "unmarked" version of the AOP report and the "marked" version of the snapshot.

REVIEWER #1:

AOP Report

Page 4, line 11. This sentence reads as if bone loss in post-menopausal women is due to the lack of mechanical loading due to microgravity. Suggest clarifying.

Agree. Revised on page 4, line 62-64 of AOP report: "Bone loss is most frequent in post-menopausal women as a result of hormonal changes and in space travelers due to the lack of mechanical loading resulting from the microgravity environment."

Page 5, line 47. Although some (but not all) animal studies may show decreased bone formation, human spaceflight studies indicate either no change in bone formation or an increase in bone formation *(see review by Stavnichuk et al., or primary research: Smith, S. M. et al. Bone metabolism and renal stone risk during International Space Station missions. 81, 712–720 (2015), Smith, S. M. et al. Men and Women in Space: Bone Loss and Kidney Stone Risk After Long-Duration Spaceflight. J. Bone Miner. Res. 29, 1639–1645 (2014).* Most animal literature demonstrating decreased formation occurred in growing animals; mature animals models suggest no change in bone formation. *E.g., Smith, B. J. et al. Skeletal Unloading and Dietary Copper Depletion Are Detrimental to Bone Quality of Mature Rats. J. Nutr. 132, 190–196 (2002).* It should be clarified here or in the Wiki that findings related to bone formation may differ based on life stage (e.g., growing or not).

Agree. The following has been added to pg 4 of the overall assessment found in the AOP snapshot:

"Human spaceflight studies indicate either no change in bone formation or an increase in bone formation (Stavnichuk et al., 2020; Smith et al. 2015, Smith, 2014)."

Most animal literature demonstrates decreased formation occurred in growing animals; mature animals models suggest no change in bone formation (Smith et al. 2002). Findings related to bone formation may differ based on life stage.

Page 7, line 32. Is there a missing word in the sentence: "exposure to therapeutic has been".

Agree. Revised to read on pg 9, line 183-185 of the AOP report: "In humans, exposure to therapeutic radiation has been demonstrated to increase the risk of bone damage as a chronic (late) effect (Willey et al., 2011)."

Page 17, line 52. "enhances osteoblasts" doesn't make sense. Sclerostin downregulates osteoblast OPG production and decreases osteoblastogenesis, not enhances it.

Agree, revised to read pg 17, line 375-376 of the AOP report: “Sclerostin inhibits osteoblasts by blocking Wnt/β-catenin signaling and enhances osteoclasts by subsequently reducing OPG”

Page 19, line 21. Suggest qualifying that current evidence “in animal models” demonstrate increased osteoclast number…

Agree. Revised to read pg 19, line 406-409 of the AOP report: “Current evidence in animal models following space-relevant stressors demonstrate increased osteoclast number or decreased osteoblast surface resulting in decreased bone formation, through measures such as the bone formation rate (BFR) and mineral apposition rate (MAR) (Dehority et al., 1999; Shahnazari et al., 2012).”

Page 18, line 39. Is it worth qualifying that most data were derived from “male” adolescent and animal models?

Agree. Revised to read pg 22, line 474 of the AOP report: “Most data in this AOP were derived from male adolescent and adult models...”

Page 21, lines 32-48. Re: bone resorption at the tissue level. The KE (2090) indicates calcium biochemistry. Human spaceflight studies typically assess calcium and phosphorus homeostasis, so would expect those findings to be included. Further, time-lapsed in-vivo micro CT imaging in animal models or high-resolution CT imaging in humans can quantify bone resorption, including eroded surface and bone resorption rate. Not sure whether studies have used these methods to evaluate resorption due to energy deposition, but the technique exists: Schulte, Friederike A et al. “In vivo micro-computed tomography allows direct three-dimensional quantification of both bone formation and bone resorption parameters using time-lapsed imaging.” *Bone* vol. 48,3 (2011): 433-42. doi:10.1016/j.bone.2010.10.007. Christen, Patrik, and Ralph Müller. “In vivo Visualisation and Quantification of Bone Resorption and Bone Formation from Time-Lapse Imaging.” *Current osteoporosis reports* vol. 15,4 (2017): 311-317. doi:10.1007/s11914-017-0372-1

Agree. We recognize that the wording is a bit unclear, although there is a measurement to quantify bone resorption, no studies were identified that met the Bradford Hill criteria that examined endpoints of bone resorption at the tissue level. We have now revised the wording to clarify this as follows in the AOP report pg 25 (line 551-554), Figure 2,: “In terms of measurements, no single study that met the Bradford Hill criteria assessed bone resorption at the tissue level. Further studies could be undertaken to examine bone resorption rate in the context of downstream KEs in the AOP by using time-lapsed micro computed-tomography (CT) imaging or advanced CT imaging in measurements.”

In the overall assessment of the snapshot pg 4: “Lastly, the bone remodeling KE includes endpoints to measure changes in the bone formation rate but has fewer endpoints to measure bone resorption. Resorption endpoints are often cell-level and are included in the altered bone cell homeostasis KE. Changes to resorption in the bone remodeling KE are determined indirectly through changes to bone formation and bone volume. Consequently, it is difficult to quantify bone resorption in the bone remodeling KE, even though it is an important contributor to bone loss. Further studies could be undertaken to examine bone resorption rate in the context of downstream KEs in the AOP by using time-lapsed micro computed-tomography (CT) imaging or advanced CT imaging in measurements.”

We recognize that there is a lack of human spaceflight studies included in this AOP. We were unable to retrieve any additional human spaceflight studies from what is already in the AOP assessing calcium and

phosphorus homeostasis that met the Bradford Hill Criteria related to the KEs. There are many human studies that look at bone health, and a few specifically look into deposition of energy induced bone loss (these are already cited). Studies which we found were primarily related to microgravity or animal studies. Below is a list of human studies cited in our AOP and associated KERs:

Increase, Cell death to Altered Bone Homeostasis and Altered Cell Differentiation Signaling to Altered Bone Cell Homeostasis: *Liu, Y. et al. (2018), "Protective Effects of α-2-Macroglobulin on Human Bone Marrow Mesenchymal Stem Cells in Radiation Injury", Molecular Medicine Reports, Vol. 18/5,*

Deposition of Energy to Altered Bone Cell Homeostasis and Deposition of Energy to Bone Loss: *Stavnichuk, M., et al. (2020), "A systematic review and meta-analysis of bone loss in space travelers", npj microgravity, Vol. 6, Nature, https://doi.org/10.1038/s41526-020-0103-2*

Deposition of Energy to Bone Loss: *Willey, J. S. et al. (2011), "Ionizing Radiation and Bone Loss: Space Exploration and Clinical Therapy Applications", Clinical Reviews in Bone and Mineral Metabolism, Vol. 9, Nature, https://doi.org/10.1007/s12018-011-9092-8.*

Nishiyama, K. et al. (1992), "Radiation osteoporosis - an assessment using single energy quantitative computed tomography", European Radiology, Vol. 2, Nature, https://doi.org/10.1007/BF00175435
Baxter N. N. et al. (2005), "Risk of Pelvic Fractures in Older Women Following Pelvic Irradiation", JAMA, Vol. 294, https://doi.org/10.1001/jama.294.20.2587

Figure 2. Are the osteoclast and osteoblast sections flipped in the "altered bone cell homeostasis" sections?

Agree, corrected.

Figure 7. Does the size of the KE in the circles have meaning? The legend indicates that size of the arrow does, but nothing is mentioned about the size of the KEs.

We have clarified in the figure legend as follows: The size of the KE circles represents relative degree of connectivity (as determined by number of connections up and downstream from the KE).

Supplementary Figure 1. Total number of records evaluated N=246 differs from what is indicated (e.g., N=107 extracted + N=75 other sources).

Agree: revised to Total number of records evaluated N = 271

AOP Wiki

As per recommendations from American Medical Association, preferred usage is "White" in place of Caucasian. Flanagin, A., Frey, T., Christiansen, S. L. & Committee, A. M. of S. Updated Guidance on the Reporting of Race and Ethnicity in Medical and Science Journals. *JAMA* 326, 621–627 (2021).

Agree. revise to read on page 2 of snapshot; "White women**..."**

Spelling: "Further efforts could be directed to developing "mthods" that are able to assess bone resorption at the tissue level."

Corrected.

In the bone remodelling KE, 2090, an example states: "increased resorption by osteoclasts and increased mineralization by osteoblasts will increase the rate of bone resorption and decrease the rate of bone formation." However, increased mineralization by osteoblasts would increase the rate of bone formation, not decrease it. Please clarify.

We have revised the sentence on page 48 of the snapshot as follows: "Disruption to this process results in an imbalance in the equilibrium of bone resorption and formation. For example, increased resorption by stimulation of osteoclast activity can lead to excessive bone resorption and subsequent weakening of bone structure. The impairment of osteoblast function and survival osteoclasts and increased mineralization by osteoblasts will increase the rate of bone resorption and decrease can decrease the rate of bone formation. These are measurable events."

We now refer to the KE as "Disrupted Bone Remodeling"

Is there a reason why human spaceflight or analog studies were not included in the relationship 2844 – altered bone cell homeostasis leads to bone remodelling. Most human spaceflight studies indicate altered bone homeostasis due to substantial increases in biomarkers of bone resorption (e.g., CTx) with minimal change or increases in biomarkers of bone formation (e.g., BSAP, P1NP, or OCN).

Human studies are included mostly in the non-adjacent KERs directly linked to the MIE (MIE to bone loss, MIE to bone cell homeostasis; MIE to Bone remodeling). This is because of the lack of statistically significant data to show a dose-concordant response to support the measurable endpoints of BFR, MAR and MS/BS in the direct relationships.

Re: 2844 – it is an important qualification that most animal studies demonstrating altered bone homeostasis as defined by an increase in osteoclast activity and decrease in osteoblast activity are in growing animals. Mature animal models tend to suggest no change or an increase in bone formation (e.g., Hui et al 2014, Wright et al 2015). If the KER evidence is based on increased bone resorption and decreased bone formation, it would appear the evidence should be higher for juvenile life stages. Or perhaps the KER evidence is based on altered bone homeostasis since increased bone resorption on its own without decreased formation can still result in decreased mineral apposition rates?

Agree. The evidence is based on increased osteoclast number/activity and decreased osteoblast number/activity leading to decreased bone formation as measured by BFR, MAR and MS/BS. From the dose- and incidence- concordance tables in KER 2844 most evidence that supports the Bradford hill criteria is using growing rodent models. We now make this clear in the biological domain of applicability pg 90 of the snapshot.

Similar comments for relationship 2845 re: why were human spaceflight or analog studies not included in the KER (e.g., Stavnichuk et al)?

See comment above. The human studies are included in the non-adjacent relationships directly linked to the MIE (MIE to bone loss, MIE to bone cell homeostasis; MIE to Bone remodeling).

Re: relationship 2849: "Short duration flights (<30 days) led to decreased bone density up to 10%, which could be due to an early onset of increased resorption and late onset of increased formation (Stavnichuk et al., 2020)." This sentence is incorrect and is not supported by the review by Stavnichuk et al. Changes in BMD are usually not detected in short duration flights.

Agree. The sentence has been removed.

Evidence assessment: KE 2090 → AO2091: the evidence supports bone loss as an imbalance between resorption and formation due to increased bone resorption. Less evidence supports a decrease in bone formation in mature animal models or in humans.

Agree. We have added the following to the KER under section "Evidence supporting the biological domain of applicability, on page 98 of the snapshot: "However, less evidence supports a decrease in bone formation in mature animal models or humans."

Evidence assessment: Known modulating factors: "Estrogen, which is lower in old age, decreases osteoclast activity and increases osteoblast activity by inhibiting the production of interleukin (IL)-6 in osteoblasts (Pacheco and Stock, 2013)." The role of osteoclasts and osteoblasts appears reversed.

Agree, revised to clarify as follows (page 11 of snapshot):
"The presence of estrogen decreases osteoclast activity and increases osteoblast activity by inhibiting the production of interleukin (IL)-6 in osteoblasts (Pacheco and Stock, 2013). As estrogen generally declines with age, older women will tend to have less estrogen, resulting in increased osteoclast activity and decreased osteoblast activity."

Re: consideration for potential applications of the AOP: "An uncertainty in the bone remodeling KE is that changes to the rate of resorption are not directly determined and are instead assumed based on changes to the bone formation rate and bone volume". It's unclear why calcium biochemistry studies were not included in the review since they are listed in the method of measurement.

Agree, we have removed this statement. As stated above although human astronaut studies measure micro-CT to assess bone loss, they do not currently measure a downstream KE in the AOP. Therefore, the studies were screened out during our scoping review. Human studies are discussed in the non-adjacent KERs linked directly to the MIE. Most human studies focus on gravitational unloading and bone loss. These studies are not relevant to the MIE unless they also examine another endpoint in our AOP.

REVIEWER #2

Is it worth referencing and discussing sibling AOPs involving energy deposition in the Introduction or Discussion to show cross-organ importance, to show how the bone AOP fits into the broader AOP context, and/or to leverage findings from these AOPs to increase weight of evidence?

a. **Specifically, AOP #483 (*learning memory effects from energy deposition*) and AOP #470 (*vascular remodeling from deposition of energy*).**
b. **Additionally, AOPs 327 through 330 (*Excessive ROS production leading to mortality*).**

Agree. Within AOP report pg 6, line 108-110 we state " This AOP is part of a larger effort to build AOPs for radiation-relevant, non-cancer diseases. Specifically, this AOP is linked through the MIE to AOP 483 (learning and memory impairment); AOP 470 (vascular remodeling) and AOP 478 (cataracts)."

We focus on the AOPs directly linked to the MIE with well-populated content and which we have authored, as there are many AOPs that are networked to our AOP that either do not have content in them or are not endorsed by the OECD.

The reader can refer to the AOP Wiki to get an understanding of the broader AOP context. In terms of leveraging the AOPs to increase the weight of evidence, we have done this for two KEs within our AOP that relate to oxidative stress and cell death. These two KEs are reused.

Page 12 and Figure 1 and Tables II and III, regarding the definitions of *Adjacent* (solid line) vs. *Non-adjacent* (dashed line) KEs, can you include a more formal definition and description of function for classification? It is not clear in the body of the manuscript, especially on page 19 (Uncertainties and Knowledge Gaps) where detailed discussions take place of adjacent KEs. Perhaps simply cutting/pasting the definitions used in the Figure 1 caption and minor additions may suffice.

Agree. We now include in Table II and Table III the following definition "Adjacent KERs demonstrate the causal relationship between two KEs. Non-adjacent KERs can be used to support the weight of evidence (WOE) of the whole AOP by bypassing KEs with less evidence."

Beginning in Section 5, page 12, the authors discuss a sequence of events following energy deposition leading to altered signaling and tissue changes that are also summarized in Figures 1, 2, and 7. However, upon review of the broader KE landscape and evidence base, it appears additional relevant KEs are neither included, nor discussed, for this particular AOP. Do additional KEs merit discussion to define the broader KE context or for future KE edits to the AOP? The below items 4) and 5) identify some of the additional KEs for consideration to be discussed.

We appreciate the reviewer's comment. We chose not to discuss other AOPs as most are under development and/or not yet endorsed by the OECD. Specifically, for radiation relevant AOPs there is only one endorsed AOP. Therefore, we only highlight the ones we have authored. This report is intended to provide a summary of the key findings related to our AOP using a specific format that is outlined by the journal.

Additionally, in the manuscript's content on Section 5, page 12, evidence from (Kondo et al. 2010) in the article's Figure 4 and 5 show a more complex timecourse than as described by this manuscript showing time-varying sequences of ROS, apoptosis, and lipid peroxidation contingent on dose of gamma-rays. As

animal studies cannot capture all data from all timepoints, we don't know when these outcomes peak. My main point is that lipid peroxidation increases from acute response (day 0 and day 3) to 10 days post-exposure for 1 Gy and 2 Gy. Hence, this type of oxidative damage marker could be the result of a secondary insult (~weeks after exposure) that is not captured in this AOP manuscript's discussion of events on page 12.

Agree. We have revised pg 16, line 333-340 of the AOP report to the state: "Additionally, rodent studies with hindlimb-unloading and radiation stressors demonstrated a dose- and time-concordant increase in ROS and a decrease in antioxidant levels accompanied by increased bone marrow cell apoptosis (Kondo et al., 2010). A complex time-course for these effects was observed, with variations in ROS, and lipid peroxidation dependent on the dose of gamma-rays. Notably, lipid peroxidation increased from the acute response (day 0 and day 3) to 10 days post-exposure for 1 Gy and 2 Gy, suggesting that oxidative damage markers can result from secondary insults occurring weeks after exposure. Therefore, time-course effects of markers of oxidative stress and follow-on detriments (e.g. lipid peroxidation) are often intricate."

Specifically for KE1392, does this KE include or interact with other key events (KEs) pertinent to free radical processes listed below, some of which are discussed on page 12 and nicely summarized/illustrated by (Nathan and Ding 2010)? In other words, are there sub-nodes or critical additional nodes that should be identified/discussed?

It is beyond the scope of this AOP to discuss all KEs that are networked to this AOP. This information is readily available in the AOP Wiki. Readers can refer to the Wiki to see how the AOP is networked to other AOPs and which KEs relate to each other.

- **Should MIE/KE 257 for ROS production be discussed?** KE 257 has no content in it, our oxidative stress KE 1392 has a section that discusses sources of ROS (direct and indirect sources).
- **Should nitrosative stress (KE1632) be mentioned with "RONS" in the manuscript?** We have added this information to KE1632
- **Should KE 177 mitochondrial dysfunction and/or KE1170 mitochondrial membrane potential be discussed?** Our scoping review did not retrieve sufficient data in the form of the Bradford Hill criteria to support inclusion of mitochondrial dysfunction or mitochondrial membrane potential as a KE in the AOP. To justify inclusion of a study it had to measure multiple endpoints representative of KEs in a dose, time, incident concordant manner or though knock-out studies. Most studies show the mitochondria to play a role in bone loss by generating ROS contributing to osteoclast activation and bone resorption, this is captured in the oxidative stress to bone cell homeostasis KER. We have included mitochondrial dysfunction in the discussion for the section related to future Potential Application of the Report pg 28-29
- **Should lipid peroxidation be discussed (KE1445)?** This is mentioned in KER 2769 we have added it to the oxidative stress KE (refer to snapshot pg 25)
- **Should oxidation to nucleic acids be discussed (KE1608 / 1634)?** This is mentioned in KER 2769, we have added it to the KE of oxidative stress as follows (refer to snapshot, pg 25)

- **Should protein oxidation products or oxphos be discussed (KE1767 / KE1477)?** This is mentioned in KER 2769, we have added it to the KE of oxidative stress, refer to snapshot pg 25

2) **Looking at downstream events, should the following KEs be discussed?**
    - **Should KE1492 (tissue resident cell activation) and KE 1494 (Leukocyte-lineage: Monocyte-Macrophage-Osteoclast) – i.e., osteoclasts fit these bills – be discussed?**
        - We have mentioned these potential KEs that could be networked to our AOP is sufficient empirical evidence is retrieved. This information can be found under the Potential Application section of AOP report pg 28-29
    - **Should KE 1493 and 2097 (pro-inflammatory mediators) be discussed?** Proinflammatory mediators will be networked to our AOP through one developed to learning and memory impairment. The following papers are not supporting the B-H criteria.
        - **Tnf and MCP-1 from (Alwood et al. 2015) to bone loss?**
          Study examines gene expression changes, and no levels of proteins are measured, no statistically significant effects seen in TNF after 2 Gy 3- and 7-days post-irradiation.
          Other markers like RANKL provide stronger concordance evidence to bone loss markers (RankL data is presented in our AOP)
        - **Tnf levels from (Little-Letsinger et al. 2021) to bone loss? –** a number of flaws identified in the study design -use of older mice, small group sizes results in very low baseline, cancellous bone mass (BV/TV) and bone turnover rates, low statistical differences. and low baseline BV/TV and turnover
        - **Tnf response (Shimizu et al. 1998)** only mRNA endpoint measurements no measurements of protein levels (see Table 1)- Gene expression changes are not essential to achieve the AO unless they have demonstrated protein level changes within the same study.

Should Nrf2 and the downstream antioxidant response element be discussed (KE 1417)? Here are some articles on Nrf2 and bone: NRF2 in context to its role as an antioxidant is discussed within the KER of oxidative stress to altered cell differentiation signaling KER. We indicate that oxidative stress in bone cells can lead to increased expression of the receptor activator of nuclear factor kappa B ligand (RANKL) and Nrf2 activation (Tahimic & Globus, 2017; Tian et al., 2017). Following activation, Nrf2 then interferes with the activation of runt-related transcription factor 2 (Runx2), and depending on the level of oxidative stress, this may result in altered bone cell function (Kook et al., 2015). NRF2 regulates the expression of antioxidant proteins that protect against oxidative damage. Oxidative stress is known to inhibit WNT signaling, a pathway crucial for osteoblast differentiation and bone formation. By reducing oxidative stress, NRF2 indirectly supports the WNT pathway and promotes bone formation.

The following papers were not added for reasons described below and there are sufficient references to support the KER

- **Review articles (Sun et al. 2015; Han et al. 2022; Che et al. 2023) to bone homeostasis?** Not radiation focused (Sun and Han studies) and Han and Che is outside scoping review time frame
- **Bone biomarkers from Nrf2-/- mice following spaceflight (Suzuki et al. 2022)** Relevant to support oxidative stress but past our scoping review timeline of 2021

- **Irradiation induced changes in Nrf2 in marrow (Liu et al. 2019) does not seem to have the necessary endpoints and (Schreurs et al. 2016)** does not measure Nrf2 protein levels. The study relates to understanding gene expression levels of pro-osteoclastogenic cytokines. Gene expression changes are not essential to achieve the AO unless they have demonstrated protein level changes within the same study.

Specific Questions on the modified Bradford Hill Criteria

The authors use a "*modified* Bradford Hill criteria" framework (Becker, et al, 2015 and illustrated in Figures 4 and 6). In Section 5, the authors state "Biological Plausibility is the highest form of evidence…". Consider adding some discussion on the definition of "highest form" to clarify this statement's intended meaning. Does this mean strongest or leading/initial form of evidence? For example, from a mechanistic point of view, this statement doesn't seem accurate if highest form is interpreted to convey the *weight* of empirical evidence. Some added description will help the reader.

It is a hierarchical system of evaluation- We have revised the sentence to state the following pg 13, line 275-278 in the AOP report: "Biological plausibility is the most strongest form of evidence in the context of the modified Bradford Hill criteria and identifies the known structural and functional aspect of each KER in the AOP, the fundamental biological understanding, and infers the biological consequence of the perturbation from the exposure/stressor."

To my reading, Bradford Hill criteria were developed for epidemiological data synthesis where, outside of clinical trials, direct testing of cause and effect in humans is not feasible. How are mechanistic interventions, especially in animals, treated/accounted for in this selected Bradford-Hill framework? The authors state that countermeasures are used for assessing attenuation of upstream KEs and downstream normal biological status (Essentiality criteria, page 17) – more procedural detail added here could be helpful to the reader. Is it worth discussing the pros and cons and limitations of the selected modified Bradford Hill criteria for the animal/mechanistic datasets included?

The OECD guidance documents provide details on the Bradford-Hill criteria and examples of types of studies that meet the criteria, this guidance document is referenced in the manuscript. We now also include the following on pg 5, line 93-103 of the AOP report:

" "This approach is used to assess causality in human, animal, and mechanistic based studies. These criteria, include biological plausibility, essentiality of KEs, empirical evidence (dose-, time-, and incidence-concordance), and the consistency of the response. By integrating diverse data types, it allows for a comprehensive evaluation of the literature. This is particularly useful in mechanistic and animal research where direct human linkages may be absent. However, challenges arise from the subjective interpretation of criteria like biological plausibility, potential discrepancies between animal models and human physiology, and difficulties in establishing clear dose-response relationships. Additionally, the emphasis on experimental evidence can be limiting when such data are scarce. Despite these limitations, the criteria remain a valuable tool for systematically evaluating causality, provided their limitations are recognized."

Comments on the Framing of the AOP #482

Much of the irradiation evidence originates from animal models. It is recommended to increase the # of human cohort studies to increase the weight and strength of evidence. An example of current and very relevant work: have the authors consulted with the lead(s) (John D. Boice, Vanderbilt) of the Million Worker Study of radiation workers (Boice 2022) to discuss skeletal outcomes? If not, this is highly recommended to consider this or other non-radiotherapy human-cohorts that includes radiation exposure (i.e., low dose, chronic exposure).

Agree, we do include human studies, they can be found within the KERs directly linked to the MIE (MIE to bone loss, MIE to bone remodeling, MIE to altered bone cell homeostasis). Most human studies do not support multiple KEs in our AOP that is the reason they are not included in the adjacent relationships. As well many human space flight studies related to bone loss discuss it in the context of gravitational unloading ex Sibonga et al., 2007. The human knowledge base related to deposition of energy leading to bone loss is limited.

In terms of the Boice et al. study, it falls outside our scoping review time-frame. We now include the following statement pg 12, line 247-248 in the AOP report: Studies published after 2021 are not included in this assessment.

<u>Structure of manuscript</u>: In terms of applicability to a broad human population, the authors *could* consider adjusting the focus of the AOP and discussion on occupational or cancer radiotherapy primarily (i.e., a relatively large-sized cohort) and have a conditional case or modulating factor for spaceflight (i.e., a small-sized cohort). Right now, these weightings seem inverted, especially considering inclusion of spaceflight data and simulated weightlessness data resulting from rodent models.

Since the prioritized prototypic stressors used to retrieve data to support the AOP relate to space environment (explained in the AOP development strategy section), the focus was on space. Human studies relevant to the adverse outcome are discussed in the introduction. We include all types of human studies: cancer, radiotherapy and space-related (see AOP report (introduction) and within the KER 2849 (Energy Deposition leads to Bone Loss).

In the introduction of bone biology, it is highly recommended that the authors introduce cancellous/trabecular versus cortical bone compartments and discuss how radiation effects may present uniquely in time, geometry, and biology in each compartment following exposure. A few references:

- **a. In rodents, trabecular effects from irradiation (Kondo et al. 2010).**
- **b. In rodents, cortical effects from irradiation are potentially more subtle and dependent on dose (Lloyd et al. 2008; Wernle et al. 2010; Oest et al. 2015; Sugimoto et al. 1991).**

Agree, we have added some content on this in the background section pg 7, line 137-141 of the AOP report as follows:

"Additionally, the two distinct bone compartments can be impacted differently from radiation exposure. The trabecular bone shows more immediate and pronounced effects post-irradiation, as evidenced in rodent studies (Kondo et al., 2010). Conversely, cortical bone effects are subtler and dose-dependent, with significant findings reported by Lloyd et al. (2008), Wernle et al. (2010), Oest et al. (2015), and Sugimoto et al. (1991)."

Consider including review articles for use in introducing topics that are well-developed in the literature:

c. **For bone cell introductions:**
    i. **ASBMSR Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 9th edition, misc chapters in Section I**
d. **For radiation effects on bone and bone cells:**
    i. **(Donaubauer et al. 2020) already in AOP report**
    ii. **ASBMR Primer 8th and 9th Primers: added to report**
e. **Mechanistic formulae to model bone turnover**
    i. **(Gerhard et al. 2009), (Boaretti et al. 2023), (Ayati et al. 2010) added to report and overall assessment**
f. **ROS/RNS/RONS and the skeletal system**
    i. **(Marques-Carvalho, Kim, and Almeida 2023; Bartell et al. 2014; Almeida et al. 2009; Manolagas and Almeida 2007; Almeida et al. 2007)**
    ii. **(Le Nihouannen et al. 2010; Agidigbi and Kim 2019).**

We have reviewed the suggested papers, and some have been added to the report and appropriate KERs. Explanations have been provided for the papers that were not added.

Marques-Carvalho et al. 2023 has been added to page 15, line 314-317 of the AOP report as follows: **"**In vitro experiments using primary osteoblasts have demonstrated that intracellular oxidative stress promotes cell death and reduces proliferation, thus explaining decreased osteoblast numbers in the bone (Marques-Carvalho et al. 2023)."

Bartell et al. 2014 and Donaubauer et al. 2020 have already been cited in the KER 'Oxidative Stress to Altered Bone Cell Homeostasis", page 106 on the snapshot as follows: "Several other papers evaluated the impact of oxidative stress on osteoclastogenesis and osteoblastogenesis and the crucial role of ROS in up and downregulation of bone resorption and deposition (Agidigbi and Kim, 2019; Bartell et al., 2014; Donaubauer, et al., 2020; Maeda et al., 2019; Manolagas et al., 2007; Tahimic and Globus, 2017).". Donaubauer et al. 2020 has also previously been discussed in the AOP report.

Almeida at al. 2009, added to page 15, line 312-314 of the AOP report as follows: "Oxidative stress can damage biomolecules, including bone cells in the marrow leading to increased death of osteoblasts and osteocytes through the induction of apoptotic signaling (**Figure 3**) (Almeida et al., 2009; Domazetovic et al., 2017; Donaubauer et al., 2020; Ott et al., 2007)"

Manolagas and Almeida 2007 was cited in our AOP but has been added as a reference to an existing sentence on page 31 line 682-684 of the AOP report "Inhibition of Wnt signaling inhibits osteoblast differentiation and activity and subsequently reduces bone formation"

Le Nihouannen et al 2010 was added into the inconsistency section of the *Oxidative stress to Altered Bone Cell Homeostasis KER* in the snapshot, on page 107 as follows: "Another study's findings show that absorbic acid treatment to RAW 264.7 cells result in significant increase in oxidative stress (H202) production. This increase in H202 concentration resulted in a significant decrease in osteoclast formation (Le Nihouann et al., 2010). This is inconsistent with other studies which show that increased levels of endpoints related to oxidative stress result in increased amounts of osteoclast production."

Agidigbi and Kim (2019) added to page 6, line 116-118 of the AOP report as follows: “Bone is a dynamic tissue that undergoes continual remodeling throughout life. This helps to maintain bone strength by replacing worn tissue with new calcified matrix (Agidigbi and Kim. 2019).”

Ayati et al., 2010, Boaretti et al., 2023 and Gerhard et al., 2009 were added to page 2 of the Overall Assessment (in snapshot) and page 9, line 181-183 of the AOP report with the following respective sentences: “Radiation-induced bone loss has also been investigated through modeling-based approaches utilizing in vivo data to comprehend the underlying mechanisms of bone turnover” and “Radiation-induced bone loss has been studied in different animals, and using in vitro cell types, modeling-based approaches to understand the mechanistic basis for bone turnover (Ayati et al., 2010; Boaretti et al., 2023; Gerhard et al., 2009).”

Wright 2018 and Willey et al. 2013 were added to the page 2 of Overall Assessment (in snapshot) and page 4, line 64-66 of the AOP report with the following respective sentences: “Growing evidence suggests that acute and chronic radiation exposure can contribute to the loss of bone mass, bone strength and changed bone quality” and “However, a growing body of evidence suggests that bone loss or changed bone quality can also result from fractionated and chronic radiation exposures, as documented by studies examining the effects of cancer radiotherapy and space radiation on humans and animals”.

Late in the manuscript - on page 20 - the authors state “Mature bones are relatively radioresistant”. Overall, however, the paper fails to differentiate *bone* as relatively radioresistant and *marrow* - the source of stem and progenitor cells for the skeleltal system – as *highly* radiosensitive. It is recommend to introduce and discuss tissue sensitivity of the integrated skeletal system and weighting factors for dose equivalent calculations (ICRP 2007) and to introduce much earlier in the manuscript.

g. **With a large bolus dose, marrow can undergo acute cell killing and subsequent repopulation or outright marrow cellularity depletion/ablation. For example, see (Turner et al. 2013), (Cao et al. 2011), (Green et al. 2012), (Kim et al. 2014), (Tatara and Monzen 2023), and (Suva et al. 1993).**
h. **Smaller doses do not induce the same level of acute cell death in marrow, hence is more easily recoverable by stem cell proliferation and differentiation (Otsuka et al. 2008) (Greenberger and Epperly 2009), though late effects are possible (Chatterjee et al. 2012), (Rastogi et al. 2012).**

Agree, this information has been added to the background section of the AOP report pg 7, line 130-136 as follows: “Mature bone tissue is relatively radioresistant compared to the highly radiosensitive bone marrow (ICRP, 2007). The bone marrow is a source of stem and progenitor cells which are essential for skeletal health. These cells exhibit significant sensitivity to radiation. Studies show that large bolus doses of radiation cause acute cell death or marrow depletion (Turner et al., 2013; Cao et al., 2011; Green et al., 2012; Kim et al., 2014; Tatara and Monzen, 2023; Suva et al., 1993) and smaller doses allow recovery through stem cell proliferation, though late effects may occur (Otsuka et al., 2008; Greenberger and Epperly, 2009; Chatterjee et al., 2012; Rastogi et al., 2012).”

Description and framing of dose levels and bounds are critical to convey to the audience of this manuscript, both in terms of spaceflight dose and radiotherapy dose – which seems to be currently missing from the manuscript. In terms of spaceflight, doses range widely from ISS missions to lunar missions and to Mars missions (Hassler et al. 2014). The data from animal studies shows a threshold dose eliciting an effect (approx. 50 cGy of heavy ions (Yumoto et al. 2010),(Alwood et al. 2017) or 100 cGy of ^{137}Cs (Kondo et al. 2009), (Alwood et al. 2012) or proton (Bandstra et al. 2008)), which also hint

at an LET effect on threshold dose. In these studies, lower doses had no effect on bone structure. Thus, how is the null hypothesis incorporated in the AOP at low dose and dose rates? At the other extreme, an upper bound of dose be introduced with appropriate conditions (e.g., focal, fractionation), with considerations for acute radiation syndrome, morbidity, and tissue necrosis/marrow ablation? In other words, bounding the dose ranges and/or energy imparted (LET) seem critical to framing the AOP properly. It is not one size fits all for *irradiation* effects on bone, which is how the manuscript currently comes across. Some potential citations:

i. **Acute dose range - (Donaubauer et al. 2020) Figure 1**
j. **Protracted / fractionated dose range – (Richardson et al. 2022)**
k. **Current mission and career dose limits for astronauts, *Standard 4.8 Space Permissible Exposure Limit for Space Flight Radiation Exposure* (NASA 2022)**

We have incorporated this information in the introduction of the report pg 4-5, line 75-83 as follows: "In terms of radiation doses, spaceflight can yield a wide range (0.4 up to 0.8 mGy/day), depending on the mission (e.g., International Space Station (ISS), lunar and Mars missions) (Hassler et al., 2014; Donaubaurer et al., 2020). Animal studies indicate threshold doses for bone effects at approximately 50 cGy for heavy ions (Yumoto et al., 2010; Alwood et al., 2017), 100 cGy of 137Cs (Kondo et al., 2009; Alwood et al., 2012), or proton exposure (Bandstra et al., 2008). Lower doses show no impact on bone structure, and at higher doses, conditions such as focal and fractionated doses become important considerations for accurate risk estimates (Donaubauer et al., 2020; Richardson et al., 2022; NASA, 2022)."

Exposure parameters related to specific studies that support the AOP can be found in the tables represented in each KER description (refer to snapshot).

For spaceflight or simulations using rodents, specifically, it is recommended that the framing be addressed throughout and especially on Modulating Factors on page 18 to include some items in the list below. Examples of articles supporting Modulating Factors for radiation, include evidence for dose response above a threshold, LET response, and osteoprogenitors proliferation and differentiation potential following radiation exposure (Alwood et al. 2012), (Alwood et al. 2017), (Bandstra et al. 2008), (Lima et al. 2017). A suggested list of Modulating Factors for irradiation response in the skeleton:

l. **Species and Strain of animal**
m. **Age at exposure**
n. **Anatomic location and compartment (cortical/cancellous) of the skeleton**
o. **Diet: dietary countermeasures against irradiation should be discussed in more detail (e.g., absence/presence of radio-protectors like phytoestrogens; omega 3; Dried Plum)**
    i. **Dietary dried plum protects against low-LET gamma and high-LET effects (Steczina et al. 2020)**
    ii. **Omega 3 vs 6 diet comparison (Little-Letsinger et al. 2021)**
p. **Additional pharmaceuticals/countermeasures should be added on page 18 and identified as a modifying factor: e.g.,**
    i. **Bisphosphonates (Willey et al. 2010), (Keenawinna et al. 2013)**
    ii. **P7C3 (Wei et al. 2023),**
    iii. **The antioxidant alpha-lipoic acid (Kondo et al. 2010) and SOD (Alwood et al. 2017)**
    iv. **PTH (Oest, Mann, et al. 2016; Oest, Gong, et al. 2016)**
q. **Exercise as a countermeasure, e.g., (Shirazi-Fard et al. 2015), (Govey, Zhang, and Donahue 2016)**

r. **Reference space-mission type (ISS mission differs from planetary orbital differs from planetary surface operations) (Hassler et al. 2014)**
s. **Total body irradiation or focal irradiation**
t. **Exposure / Fractionation schedule**
u. **Dose rates**
v. **Radiation type and quality**
    i. **Linear energy transfer LET (Durante and Cucinotta 2011)**
    ii. **Track structure (Plante and Cucinotta 2008)**

The modulating factors listed above not included are a study by Little-Letsinger et al., 2021, which does not support adjacent KERs in our AOP. The countermeasure by Shirazi-Fard et al., 2015 and Govey, Zhang, and Donahue, 2016 were microgravity related and does not support our AOP. Wei et al., 2023 is past 2021 and outside of our review timeframe.

The modulating factors are all discussed within each of the KER descriptions which can be found in the snapshot documents and within the AOP Wiki. We now also incorporate these within the AOP report on pg 22-23, as follows:

"Some prominent modulators of the AOP include the species and strain of the animal, and the specific anatomical location and compartments (cortical versus cancellous) of the skeleton. The type of space mission, whether ISS, planetary orbital, or planetary surface operations can also influence the progression to the AO (Hassler et al., 2014). Additionally, factors such as total body versus focal irradiation, exposure/fractionation schedule, dose rates, and radiation type and quality, including track structure (Durante and Cucinotta, 2011; Plante and Cucinotta, 2008), are pivotal in shaping the radiation response in the skeleton.

Most data in this AOP were derived from male adolescent and adult models with a moderate to high level of evidence, while there were fewer studies using preadolescent models. Certain KEs showed age to modulate the loss in bone mass and quality after irradiation, however there was inconsistency in findings (Willey et al., 2011). Elderly individuals typically have reduced estrogen levels. Estrogen decreases osteoclast activity and increases osteoblast activity, possibly through the inhibition of IL-6 production (osteoclast stimulatory molecule), thereby reducing excessive bone resorption. Therefore, the damaging effects of radiation on bone are compounded by age-associated reduced estrogen levels (Pacheco & Stock, 2013). As well, there was a weak association of cortical bone loss and osteoblast dysfunction during spaceflight in rats from age 6 to 20 weeks (Fu et al., 2021). Overall, the effect of age on bone loss due to space exposure requires further investigation (Fu et al., 2021; Moussa et al., 2022).

The AOP can also be influenced by other types of modulating factors that act on oxidative stress mechanisms and bone remodeling. Dietary countermeasures such as dried plums protect against radiation induced radical formation (Steczina et al., 2020; Schreurs et al. 2016). Pharmaceuticals, such as bisphosphonates (Willey et al., 2010; Keenawinna et al., 2013), the antioxidant alpha-lipoic acid, human catalase, and superoxide dismutase (Kondo et al., 2010; Alwood et al., 2017; Schreurs et al. 2020), scavenge oxidative stress events. The parathyroid hormone (Oest, Mann, et al., 2016; Oest, Gong, et al., 2016) has been shown to alter bone cell homeostasis. Additionally, a few osteoclast inhibitors, and osteoblast activators, have various effects on bone remodeling activity and ultimately bone loss (Chandra et al., 2014; Huang et al., 2019; Li et al., 2020a; Li et al., 2020b; Liu et al., 2018; Lloyd et al., 2015; Willey et al., 2010; Yang et al., 2019; Zhang et al., 2020). Mutations in the SOST gene for sclerostin also was shown

to modulate the bone remodeling leading to bone loss (Chandra et al., 2017). Lastly, zoledronic acid binds strongly to hydroxyapatite in bone, and has been shown to prevent bone loss in various measurements (increased BV/TV, Tb.N and Tb.Th) through its action on osteoclast-mediated bone resorption (Keenawinna et al. 2013)."

Additional Evidence to consider for inclusion within the AOP

Discussion of irradiation and mitochondrial role/function in the skeletal system are not included. The following articles offer two examples:

- **w. A transgenic mouse study which expresses catalase antioxidant in mitochondria (mCAT) and treating mouse with combined unloading and irradiation to mimic spaceflight conditions – fundamental to free radical hypothesis (Schreurs et al. 2020). The animals still experience bone loss.**
- **x. Article investigating fractionated irradiation and sirt-3 in osteocytes (Richardson et al. 2022).**

Agree. We have added this information to the AOP report under the section titled "Potential Application" as described at the end of this section (pg 28-29 of report) .

This AOP as a living document could evolve to include some additional KEs. Emerging studies highlight the importance of mitochondrial catalase (Schreurs et al., 2020; Richardson et al., 2022), and processes such as cellular senescence to promote bone loss (Coppe et al., 2010; d'Adda di Fagagna, 2024; Kumar et al., 2021). Networking existing KEs in the AOP Wiki could also be considered following identification of specific empirical data for understanding osteoclast-mediated bone resorption. This includes: leukocyte recruitment/activation (KE 1494); tissue resident cell activation (KE 1492) and pro-inflammatory mediators (KE 1493; 2097). Evidence highlights the role of pro-inflammatory mediators such as TNF, MCP-1, IL-1beta, and IL-6 in promoting osteoclast activity (Alwood et al. (2015), Willey et al. (2011), Little-Letsinger et al. (2021), and Shimizu et al. (1998) but would need to be assessed at the protein-level in the context of bone loss. Additionally, the phenomenon of abscopal effects, where bone loss occurs outside the irradiated area (Burr et al. 2010, Jia et al., 2011; Wright et al., 2015), and bone matrix changes due to Advanced Glycation End-Products (AGEs) (Pendleton et al., 2021; Mandair et al., 2020; Gong et al., 2013; Oest and Damron, 2014; Oest, Gong, et al., 2016), are areas for future study.

Cell death pathways, including apoptosis, necrosis, and autophagy are discussed on page 15, presumably primarily for dividing stem and progenitor/precursor cells in the marrow and also for differentiated cells in the osteoblast/osteocyte lineage. It does not appear that *senescence* is mentioned in the manuscript body (though at least 15 titles of references state *senescence*); A thorough discussion of Senescence Associated Secretory Phenotype (SASP) is neither introduced, nor developed, yet literature shows SASP, especially from *in vitro* studies to play a role in the cellular irradiation response at high doses, pertinent to tissue aging (Coppe et al. 2010; d'Adda di Fagagna 2024) (Kumar et al. 2022). *In vitro* work can be specific to osteocytes (Wang et al. 2021) and other bone-related cell types, including marrow HSCs (Wang et al. 2006) and MSCs (Bai et al. 2020).

Agree. We have cited three of these papers under the section titled "Potential Application". Cell senescence could be explored in future iterations of the AOP. Pg 28-29
Coppe et al 2010- is not specific to bone
D'adda et al, 2024- past our scoping review date of 2021
Kumar et al. 2022-past our scoping review date of 2021

Wang et al. 2021; 2006- only measures cell senescence no additional endpoints measured that relate to our AOP
Bai 2020-relevent, cited

Irradiation effects on bone cell replenishment from marrow precursors or progenitors is not specifically introduced/discussed. The following articles should be reviewed for potential inclusion:

- **Effects of radiation dose and LET on growth potential of marrow after irradiation**
  - **Stem cell pool depletion (Green et al. 2013 ; Green and Rubin 2014-** review on radiation effects on bone marrow**; Green et al. 2012-r**adiation on bone marrow, endpoints for bone loss is measured**) and oxidative damage (Cao et al. 2011)-**
  - **Oxidative stress and alpha-lipoic acid Kondo et al 2010):** reports apoptosis of bone marrow cells**) and SOD** (Alwood et al. 2017 - gene data not support BH criteria, bone loss is measured) countermeasures for low-LET gamma irradiation presented**)**
  - **Short-term effects following high-LET particulate irradiation (Yumoto et al. 2010) –** measures bone loss and bone cell homeostasis (non-adjacent KER) and supports bone replenishment damage (added paper)
  - **Dietary dried plum countermeasure against low-LET gamma or high-LET particulate irradiation (Steczina et al. 2020) –** measured bone loss and diet plum as countermeasure however this paper does not discuss a mechanism on how dietary plum can promote bone cell replenishment. Not sufficient evidence to support concept of bone replenishment.

Agree. We have added this information to the AOP report under the background section of the AOP report as follows pg 7, line 145-149:

"The accepted understanding of bone loss involves the disturbance of homeostatic conditions in the bone environment, particularly of bone cells that originate from the bone marrow (Bilezikian et al., 2018). A critical aspect of bone health is bone cell replenishment (Green et al., 2012; Green et al., 2013), ensuring the balance of proliferation and differentiation of osteoblasts and osteoclasts (Green and Rubin, 2014).

The following has been added to Summary of scientific Evidence Section of AOP report on pg15-16, line 317-321:

Particularly at high doses (>2Gy) of radiation (e.g., heavy ions) the marrow cell population of the bone can be damaged/reduced in number through apoptosis (Yumoto et al., 2010; Cao et al; 2011). Bone morrow cells can be protected from damage with the addition of antioxidants (e.g. superoxide dismutase, dried plum extracts) (Steczina et al., 2020; Alwood et al., 2017).

Acute and transient *stimulatory* effects of osteoblast lining cells has been found – this subtle evidence is not introduced in background or discussed in other sections (e.g., page 16 histological sections):

i. **Acute stimulation of osteoblast lining cells strongly correlated with marrow depletion (Turner et al. 2013).**
ii. **10 cGy stimulation of osteoblast lining cells (Karim and Judex 2014).**
iii. **Apparently positive structural effects of low-LET irradiation (Bokhari et al. 2019).**

Thank you for highlighting these inconsistencies in data in context of our AOP. We have added the following studies to the "uncertainties and inconsistencies" sections of relevant KERs:

Turner et al. 2013 has been added to the KER of "Deposition of energy to Altered Bone cell homeostasis" with the following sentence: "Mice receiving 6 Gy of radiation showed a significant increase in the osteoblasts and osteoclast-lined bone perimeter, as opposed to a decrease in osteoblast number after a high dose of radiation (Turner et al., 2013)".

Karim and Judex, 2014 was added to the KER of "Deposition of energy to bone loss" with the following sentence: "There was a significant increase in trabecular BV/TV, Conn.D and Tb.N after mice were exposed to 4.4 cGy of ionizing radiation (Karim and Judex, 2014)". The study was also added to the "Bone remodeling to Bone loss" KER with the following sentence: "Mice exposed to 4.4cGy experienced a significant increase in trabecular BV/TV and significant decrease in the SMI compared to the non-irradiated controls, contrary to the expected outcomes of decreased BV/TV and increased SMI (Karim and Judex, 2014)".

Bokhari et al. 2019 has been added to the "Deposition of energy to bone loss" KER with the following description: "There was approximately a 2-fold increase in %BV/TV of the distal femur of mice following a 0.5 Gy of 56Fe compared to the sham-irradiated group (Bokhari et al., 2019)".

Willey et al. 2010 was added to the "Deposition of energy to bone remodeling" KER with the following sentence: "The MAR of the proximal tibial metaphysis from mice irradiated with 2 Gy X-rays showed significant increase after 1 week as opposed to a decrease (Willey et al., 2010)".

We have also highlighted the studies within our AOP report on page 25, line 546-550 with the following sentence: "It was also observed that radioadaptive effects occurred when low doses of radiation were applied to subjects, such as acute and transient stimulatory effects in osteoblasts lining cells. The directionality of the events was opposite to the expected adverse effects when exposed to high doses of radiation (Bokhari et al., 2019; Karim and Judex, 2014; Turner et al., 2013; Willey et al., 2010)".

Irradiation-induced increase in mineral apposition in cancellous tissue of the tibial metaphysis (Willey et al. 2010).

The aspect is discussed in the "Deposition of energy to altered bone cell homeostasis", "Deposition of energy to bone loss" and "Bone remodeling to bone loss" and "Deposition of energy to bone remodeling" KER.

The full temporal complexity of responses in osteoclast cells is not captured in the background or later section discussions (e.g., page 16 histological discussions). In my view, shortly after irradiation exposure, there is an acute co-stimulation of osteoclasts along RANKL-induced and inflammatory pathways, yet, oppositely, there is indication of late inhibition of osteoclast differentiation and bone resorption:

- **For radiotherapy doses, (Oest et al. 2015) is cited, but needs more discussion to capture the temporal effects of # of osteoclasts in the distal femoral metaphysis and epiphysis and late cortical thickening. Additional work from this group (Wernle et al. 2010) shows bone structure outcomes showing temporal variation for cancellous and cortical in distal femur.**

  MIE to bone loss- temporal and dose response- 12 week loss of bone stregnth following 5- or 20 Gy radiation

- **(Zhai et al. 2019) reports varying osteoclastogenic potential based on single or fractionated radiotherapy doses.**
  Study is relevant shows complexity in the time-response effect
- **(Alwood et al. 2015) reports temporal gene expression of osteoclastogenic and inflammatory factors in marrow following irradiation.**
  Measures gene expression, gene expression data not used to support AOP due to not be able to show the essentiality of the response to downstream effect, needs protein data
- **(Willey et al. 2011) reports temporal osteoclast and inflammation markers following irradiation.**
  Review article discusses time to bone loss following radiation (already included in AOP report),
- **(Wei et al. 2023) shows countermeasure against osteoclast induced bone resorption. signaling to homeostasis -**outside review time-frame

Thank you for the suggestion, we have reviewed the studies and have added the relevant ones and expanded on the temporal understanding of the AOP. The Wei et al. 2023 study is outside of the review time frame and therefore not included in our AOP. This content has been added to the AOP report pg 20, line 425-435:

"The temporal responses of deposition of energy to bone loss have not been examined across all KEs in a single study. Most data examines the time from radiation to bone loss. Collectively, studies show that the temporal response are not clear, and the type of radiation and the timing of measurements can affect osteoclastogenic potential and bone cell homeostasis. In terms of bone loss, radiotherapy studies show there is significant complexity in time-response effects. Recent clinical studies observed that irradiation leads to an early increase in osteoclast number and activity, marked by elevated serum TRAP5b levels within 24 hours, and significant bone loss within a week (Zhai et al. 2019; Willey et al. 2011). This can vary depending on whether radiotherapy doses are administered as a single dose or in a fractionated manner. Studies do consistently show that osteoclast number correlates temporally with trabecular resorption (Oest et al., 2015).

To the overall assessment, pg 4 of the snapshot:

The temporal responses of energy deposition to bone loss have not been comprehensively examined across all KEs in a single study, with most data focusing on the interval from radiation exposure to bone loss. It is well accepted that deposition of energy occurs immediately following irradiation, and downstream changes will always occur later in a time course. The subsequent radical formation occurs within microseconds (Azzam, Jay-Gerin, and Pain, 2012), and studies have observed the resulting oxidative stress as early as 2 minutes post-irradiation (Wortel et al., 2019). Altered cell differentiation signaling is a molecular-level KE like oxidative stress, and both KEs occur with a similar time course, making the assessment of time concordance difficult between these KEs. However, oxidative stress can still be observed slightly earlier than altered cell differentiation signaling (Wortel et al., 2019). The ensuing cell death due to oxidative stress often occurs within days post-irradiation, while altered bone cell homeostasis owing to both altered cell differentiation signaling and cell death is subsequently observed about a week after irradiation (Liu et al., 2018). Then, from multiple weeks to a month post-irradiation, bone remodeling is observed to favor resorption over formation (Alwood et al., 2010; Chandra et al., 2017; Chandra et al., 2014; Zhai et al., 2019). The resulting bone loss presents after this, with the greatest bone loss and risk of fractures observed months to years following irradiation (Holm et al., 1996; Nishiyama et al., 1992; Oest et al., 2018; Zou et al., 2016). However, in animal models studies consistently show that irradiation leads

to an early increase in osteoclast number and activity, marked by elevated serum TRAP5b levels within 24 hours and significant bone loss within a week, although this response can vary based on the type and administration of radiotherapy (Zhai et al. 2019; Willey et al. 2011; Oest et al. 2015).

We have also added some additional content to relevant KERs as follows:
Oest et al. 2015 was added to MIE to altered bone cell homeostasis (temporal concordance section), page 117 of snapshot: "Oest et al. (2015) observed increased osteoclast numbers correlated temporally with trabecular resorption, most pronounced 2 weeks post-irradiation (5 Gy and 4X5Gy of X-rays)".

Irradiation effects on osteocytes are not discussed in depth. The first mention of osteocytes is not in the abstract, but rather in the middle of page 6. The following articles could be considered for inclusion to deepen this important area of knowledge:

Agree, studies that discuss the irradiation effects observed on osteocytes are included in the AOP report pg 9-10, line 189-201 as follows:

For example, high doses of focal electron irradiation in rabbits reveal complex temporal effects on bone tissue, including alterations in tissue-level integrity, marrow and osteocyte viability, and vascularity (Sugimoto et al., 1991; 1993; Takahashi et al., 1994). Combined partial weight-bearing and 28Si irradiation can also lead to the induction of osteocytes, contributing to a reduction in bone formation (Macias et al. 2016). There is also evidence suggesting osteolysis and subsequent bone loss following spaceflight (Blaber et al., 2013) and simulated weightlessness (Lloyd et al., 2014). It is important to note that in these studies, one cannot rule out the potential combined effect of ionizing radiation and microgravity. In terms of osteoblasts, several studies show the reduction and impairment in osteoblast progenitors and mature osteoblasts compromise bone formation and contribute to bone loss (Sawajiri et al. 2003; Chandra et al. 2017; Jacobson et al. 2010). Acute doses of iron ions as low as 0.1 Gy (Yumoto et al., 2010) or protons as low as 1 Gy (Lloyd et al., 2012) also cause loss in bone volume within one week, in animal models (Willey et al., 2011; Zhang et al., 2018a).

Adipocyte infiltration into the marrow is not discussed, though much evidence exists (Ewing 1926; Bond, Fliedner, and Archambeau 1965) (Guerra et al. 2018; Costa and Reagan 2019; Nakashima et al. 2024), (Wei et al. 2023), (Chandra et al. 2017). Adipocytes could contribute to the pro-inflammatory milieu in marrow following irradiation.

Agree. The following has been added on page 7, line 141-144 of the AOP Report.

"Furthermore, after irradiation, studies have shown that adipocytes are rapidly infiltrated into the bone marrow, causing a significant decrease in volume for both the trabecular bone and the total bone (Costa and Reagan. 2019, Guerra et al. 2018, Nakashima et al. 2024)."

Page 17: *In vivo* studies of abscopal effects - where bone loss is observed outside of the irradiated area - are few in #, but mechanistically are important to acknowledge. At least one uncited article shows focal irradiation of the gut having effects on bone (Jia et al. 2011). Another shows effects in a contralateral, unirradiated limb (Wright et al. 2015). EG Wright's work shows macrophages as critical links in abscopal effects in marrow (Rastogi et al. 2012), (Burr et al. 2010).

Agree. Abscopal effects are discussed in the KER of "MIE to bone loss" under essentiality. We also include it within the AOP report under 'Potential applications" on page 29, line 625-628 as follows: "Additionally, the phenomenon of abscopal effects, where bone loss occurs outside the irradiated area (Burr et al. 2010, Jia et al., 2011; Wright et al., 2015), and bone matrix changes due to Advanced Glycation End-Products (AGEs) (Pendleton et al., 2021; Mandair et al., 2020; Gong et al., 2013; Oest and Damron, 2014; Oest, Gong, et al., 2016), are areas for future study."

Bone matrix changes related to radiation and oxidative stress, like Advanced Glycation End-Products (AGEs) are not discussed, which could fit under ROS of protein oxidation KEs 257 and 1767. Some examples of relevant articles include:
(Pendleton et al. 2021; Mandair et al. 2020; Gong et al. 2013; Oest and Damron 2014; Oest, Gong, et al. 2016)

Agree. We have added this AGE information to the KE of oxidative stress and also within the AOP report as an area for future exploration refer to page 28-29:

This AOP as a living document could evolve to include some additional KEs. Emerging studies highlight the importance of mitochondrial catalase (Schreurs et al., 2020; Richardson et al., 2022), and processes such as cellular senescence to promote bone loss (Coppe et al., 2010; d'Adda di Fagagna, 2024; Kumar et al., 2021). Networking existing KEs in the AOP Wiki could also be considered following identification of specific empirical data for understanding osteoclast-mediated bone resorption. This includes: leukocyte recruitment/activation (KE 1494); tissue resident cell activation (KE 1492) and pro-inflammatory mediators (KE 1493; 2097). Evidence highlights the role of pro-inflammatory mediators such as TNF, MCP-1, IL-1beta, and IL-6 in promoting osteoclast activity (Alwood et al. (2015), Willey et al. (2011), Little-Letsinger et al. (2021), and Shimizu et al. (1998) but would need to be assessed at the protein-level in the context of bone loss. Additionally, the phenomenon of abscopal effects, where bone loss occurs outside the irradiated area (Burr et al. 2010, Jia et al., 2011; Wright et al., 2015), and bone matrix changes due to Advanced Glycation End-Products (AGEs) (Pendleton et al., 2021; Mandair et al., 2020; Gong et al., 2013; Oest and Damron, 2014; Oest, Gong, et al., 2016), are areas for future study.

<u>Minor suggestions for edits</u>

Page 5, Background: change "constant remodeling" to "continual remodeling", as remodeling rate is always present, but not a constant rate.

The following has been changed to "Bone is a dynamic tissue that undergoes continual remodeling throughout life". On page 6, line 116 of the AOP report.

Page 6, "temporary anatomical structures" could cite (Jilka 2003)
This reference has been added to the AOP report on page 8, line 154.

Page 7, use of "evolve" to describe osteocytes. It's recommended to change this to "terminally differentiate"

The sentence has been changed to “With time, osteoblasts become trapped in the bone matrix and terminally differentiate into osteocytes” on page 9, line 176-177 of the AOP report.

Page 13, clause “through upregulation of RANK-L production in osteoblasts” could be appended with “osteoblast-lineage cells” as osteocytes should not be excluded.

The sentence has been changed to “These molecules can activate osteoclasts directly as well as indirectly through the upregulation of RANK-L production in osteoblast-lineage cells”. On page 16, line 343-345 of the AOP report.

Page 14, clause “and enhances osteoblasts by subsequently reducing OPG” should likely be “osteoclasts”.

Sentence revised to “Sclerostin inhibits osteoblasts by blocking Wnt/β-catenin signaling and enhances osteoclasts by subsequently reducing OPG” pg17, line 375-376 of the AOP report.

Page 15, regarding the clause “Due to inability of scavengers (phagocytes) to reach osteocytes”, isn’t this one function of osteoclastic bone resorption, especially in trabecular region and near cortical surfaces? Consider modifying this clause for accuracy.

The sentence reads as follows: “If apoptotic cells are not engulfed by phagocytes, necrosis occurs with the rupture of the plasma membrane. This releases immunostimulatory molecules, such as high mobility group box 1 (HMGB1), and eventually the inflammatory cytokines IL-6, TNF-α and RANK-L (Jilka et al., 2013; Komori, 2013).” pg18, line 384-387 of the AOP report.

Page 16, authors use “dysregulated bone remodeling” clause twice in this paragraph. Please review for revision.

The sentence has been changed to “Disruption of bone remodeling…” on pg 19, line 416-417 of the AOP report.

Page 16, clause “promoting bone matrix resorption back into the bloodstream” is somewhat confusing (and also used in following sentence). Consider “bone matrix transport” instead of resorption.

The following has been changed to “Disruption of bone remodeling can cause bone loss by either promoting bone matrix transport back into the bloodstream to support vital functions or by reducing bone matrix deposition.” pg19, line 416-418 of the AOP report.

Page 18, check logical use of “and” in lists of antioxidants and lists of transgenic animals. An “or” clause may be more appropriate.

Sentence revised to “To assess essentiality of oxidative stress, several studies treated their models with antioxidants, such as N-acetyl cysteine, Amifostine, cerium (IV) oxide, or curcumin (Huang et al., 2019; Kook et al., 2015; Wang et al., 2016; Xin et al., 2015).” pg21, line 452-455 of the AOP report.

Page 24, Potential Applications, the leading sentence “The present qualitative AOP was developed…” is important and frames the organization of everything prior. Can varieties of this sentence be inserted in additional places like the abstract and sections 1 and/or 3? It feels buried at this location.

Added it to the abstract as follows: “This qualitative AOP was developed in collaboration with bone loss research experts to aggregate relevant findings, supporting ongoing efforts to understand and mitigate human system risks associated with radiation exposures.” pg3, line 44-46 of the AOP report.

Page 25, clause “risk assessment strategies”, consider citing (Orwoll et al. 2013).

Sentence has been revised to “The ultimate goal is to develop quantitative AOP that could be used to inform risk assessment strategies for space travel and other areas such as cancer radiotherapy (Orwoll et al. 2013)” pg29, line 637-639 of the AOP report.

Page 66, Figure 2, “Tissue” box: the clause “shift to rod-like geometry”, consider changing to “more rod-like” and that is only for trabecular tissue (see comment on cortical vs cancellous compartments above).
Sentence revised to “The resulting shift in bone remodeling towards increased resorption is highlighted by the degradation of plate-like trabeculae into the weaker, more rod-like trabeculae (measured by SMI) “ pg44 of the AOP report.

Citations in this review – for cross referencing and for consideration of inclusion in the “Collection of Evidence for AOP Building”

We have incorporated the relevant citations into the AOP Report specifically within the background section or areas for future direction of AOPs in context of skeletal health. Those studies meeting Bradford hill criteria were further added to the appropriate KERs. Refer to Table for details on studies that are included and location.

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